Molecular Microbiology (2003) 48(5), 1275–1288 doi:10.1046/j.1365-2958.2003.03521.x

Identification of *Candida albicans* genes induced during thrush offers insight into pathogenesis

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Summary

Candida albicans **causes a wide spectrum of diseases, ranging from mucocutaneous infections like oral thrush to disseminated candidiasis. Screening for *C. albicans* genes expressed within infected hosts might advance understanding of candidal pathogenesis, but is impractical using existing techniques. In this study, we used an antibody-based strategy to identify *C. albicans* genes expressed during thrush. We adsorbed sera from HIV-infected patients with thrush against candidal cells grown *in vitro* and screened a *C. albicans* genomic expression library. We identified 10 genes encoding immunogenic antigens and used reverse transcription-polymerase chain reaction to verify that they were induced within thrush pseudomembranes recovered from a patient. The *in vivo* induced genes are involved in diverse functions, including regulation of yeast-hyphal morphogenesis, adhesion to host cells, nutrient uptake, phospholipid biosynthesis and amino acid catabolism. Four genes encode known virulence determinants (*HWP1*, *CST20*, *CPP1* and *RBF1*). Another gene, *LPD1*, for which a role in candidal pathogenesis is unknown, encodes a protein homologous to a bacterial virulence determinant. Most importantly, disruption of *CaNOT5*, a newly identified gene, conferred defects in morphogenesis, decreased adherence to human buccal epithelial cells and attenuated mortality during murine disseminated candidiasis, proving that our strategy can identify genes encoding novel virulence determinants.**

Introduction

The fungus *Candida albicans* is a harmless colonizer of mucosal surfaces in most healthy humans. In immunocompromised individuals, however, *C. albicans* is able to cause a diverse range of mucosal and systemic infections, including acute pseudomembranous oral candidiasis (thrush), which is the most common opportunistic infection of HIV-infected patients, and disseminated candidiasis, which carries mortality rates of approximately 40% (Nguyen *et al.*, 1996). Clearly, host immune function is a major determinant of candidal pathogenesis. The specific mechanisms by which the organism might sense disturbances in its normal commensal relationship with the host and transform itself into an opportunistic pathogen, on the other hand, are not well understood. Although disruptions of *C. albicans* genes encoding adhesion proteins, secreted hydrolytic enzymes, iron permeases and regulators of cell morphogenesis attenuate mortality in animals with disseminated candidiasis, no individual gene encodes a dominant determinant of candidal virulence (Calderone and Fonzi, 2001). Rather, pathogenesis likely depends upon the organism efficiently adapting to changes in the local host environment by co-ordinating the expression of multiple genes (Mahan *et al.*, 2000; Staib *et al.*, 2000). For this reason, a more complete understanding of candidal pathogenesis will require defining the roles of individual genes encoding virulence determinants, as well as the larger patterns of *C. albicans* gene expression within the infected host (Mahan *et al.*, 2000).

In the past decade, several *in vivo* expression technologies have been developed to detect the expression of bacterial genes during animal infections (Mahan *et al.*, 2000). Novel applications of these technologies have dramatically expanded the understanding of the mechanisms

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of bacterial pathogenesis. It is now clear, for example, that expression patterns of virulence-associated genes observed *in vitro* often do not correlate with the expression during actual infections (Mahan *et al.*, 2000). Investigators have used these *in vivo* expression technologies as screening tools to identify novel genes that are preferentially expressed within animals compared to during routine growth in the laboratory (Mahan *et al.*, 1993). Selected *in vivo* induced genes identified by these screening strategies have been demonstrated to encode previously unrecognized virulence determinants, including targets for effective new vaccine and therapeutic strategies (Mahan *et al.*, 1993; McKenney *et al.*, 1999).

It is reasonable to assume that *in vivo* expression technologies will also advance the understanding of candidal pathogenesis. Indeed, existing technologies have been adapted to define differential expression patterns of several *C. albicans* genes known to be associated with virulence during murine candidiasis (Staib *et al.*, 2000; 2002). Unfortunately, screening strategies to identify *C. albicans* genes expressed during the infectious process are impractical due to technical limitations resulting from the organism's diploid genome and asexual life cycle (Cormack *et al.*, 1999; Goldman, 1999). To date, only a handful of *C. albicans* genes that are induced during oral candidiasis in rats have been identified (Zhao *et al.*, 1998). The contributions of these genes to the pathogenic process have not been investigated, nor have the larger patterns of candidal gene expression *in vivo* been defined.

The purpose of the present study was to adapt an antibody-based screening strategy called *In Vivo* Induced Antigen Technology (IVIAT) (Handfield *et al.*, 2000) to identify *C. albicans* genes expressed within the human host during thrush. If this approach was proven feasible, we sought to demonstrate that a subset of the *in vivo* induced genes encode candidal virulence determinants.

Results

Screening a C. albicans *genomic expression library with adsorbed sera recovered from HIV-infected patients with active thrush*

We pooled sera from 24 HIV-infected patients with thrush, in order to include a broad range of anticandidal antibodies. The patients represented typical clinical presentations in the era of highly active antiretroviral therapy (Table 1). The pooled sera underwent repeated rounds of adsorption against whole cells and French-pressed cell extracts of a clinical *C. albicans* strain (Ca 172) in order to remove the antibodies that were reactive with proteins made by *C. albicans in vitro*. The successive rounds of adsorption against the whole cells resulted in a substantial reduction in the anticandidal antibody titre, with most of the reduction noted after the first round (OD_{495} decreased from 0.9 preadsorption to 0.4 and 0.1 after one and five rounds of adsorption, respectively). Adsorption against epitopes exposed by cell lysis and heat denaturation resulted in a further diminution of the titre (OD_{495} was <0.06 after the last adsorption step).

Table 1. Demographics and characteristics of 24 HIV-infected patients with thrush.

Median age in years (range)	37 (29–56)
Male sex	92% (22/24)
Median CD4 (range)/mm^3	58 (16–205)
Median viral load (range) ml^{-1} blood	189 000 (49,000- ≥ 750 000)
Anti-retroviral therapy:	17% (4/24)
NRTI[a]	4
NNRTI[b]	2
Protease inhibitor	2
Median duration of symptoms prior to diagnosis of thrush (range)	1 month (3 weeks-2 months)
Thrush presenting at time of HIV diagnosis	4% (1/24)
Concomitant esophageal candidiasis	8% (2/24)
Thrush refractory to systemic antifungal therapy	4% (1/24)

a. NRTI, nucleoside reverse transcriptase inhibitor.
b. NNRTI, non-nucleoside reverse transcriptase inhibitor.

We used the adsorbed sera to screen approximately 21 000 colonies of a *C. albicans* genomic expression library in *E. coli,* and detected colonies expressing immunogenic antigens by reaction with antihuman immunoglobulin (Cappel/ICN). Among 32 signals potentially conforming to reactive colonies during the first round of screening, 12 clones were confirmed to be reactive during a second round of screening. Eleven of these remained reactive after the third and fourth rounds of screening, and were taken to represent true positives. Recombinant plasmids from the clones were purified, the cloned inserts sequenced using pET30 primers (Novagen), and potential open reading frames (ORF) identified (ExPASy, Swiss Institute of Bioinformatics). The 11 clones contained 10 unique DNA sequences; one pair of clones contained identical DNA sequences. Eight of the unique DNA sequences encoded single ORFs (size of inserts: 589–1117 basepairs), and two sequences encoded two or more potential ORFs. For the two clones containing sequences that encoded multiple potential ORFs, we subcloned each ORF into pET30 and rescreened with the adsorbed sera. In each case, a single ORF was responsible for reactivity (size of ORFs: 184 and 474 basepairs). Therefore, 10 ORFs expressing antigens reactive with the adsorbed sera were identified, ranging in size from 184 to 1117 basepairs (Table 2). Analysis by SDS gel electrophoresis and Western analysis using the adsorbed sera confirmed that the sizes of the immunogenic antigens

Table 2. Descriptions of *C. albicans* genes identified by IVIAT screening.

IVIAT clone	Genomic location of reactive ORF[a]; genomic location of the complete gene[a]	*C. albicans* gene: description of protein encoded by gene	Relevance to candidal pathogenesis
CH7	Contig 6–2372 (10150–11014); (9583–11487)	*HWP1* (hyphal wall protein): hyphal surface mannoprotein that is a mammalian transglutaminase substrate.	Disrupted mutant exhibits defective hyphal formation, reduced adherence to buccal epithelial cells and reduced injury of endothelial cells. In a murine model of systemic candidiasis, it is normal in initiating infection but deficient in maintaining infection (Staab *et al.*, 1999)
CH3	6–2466 (17834–18950); (16039–19725)	*CST20*: protein kinase that is a component of the mitogen-associated protein kinase (MAPK) pathway regulating morphogenesis.	Disrupted mutant is defective in the ability to form hyphae and is less virulent in a murine model of systemic candidiasis (Kohler and Fink, 1996).
CH9	6–2491 (3164–3752); (3017–4813)	*CPP1*: protein phosphatase that negatively regulates MAPK pathway.	Disrupted mutant exhibits hyperfilamentous morphology and is less virulent in murine models of systemic candidiasis and candidal mastitis (Csank *et al.*, 1997; Guhad *et al.*, 1998)
MA1	6–2315 (17868–18051); (17868–19427)	*RBF1* (RPG-box binding factor): putative transcription factor that represses hyphal formation.	Disrupted mutant exhibits hyperfilamentous morphology and is less virulent in a murine model of systemic candidiasis (Ishii *et al.*, 1997)
MA2	6–2466 (34740–35213); (34727–36136)	*PTH1* (proline transporter helper): involved in gluconate transport.	Not yet elucidated.
KHOI	6–1922 (2109–2839); (1197–3731)	*CDC24*: GTP/GDP exchange factor for the signal transducer Cdc42p[b]	Not yet elucidated.
CLEM	6–2267 (2462–3112); (2410–3783)	*PCT1* (choline-phosphate cytidylyl transferase): enzyme of the CDP-choline pathway for phosphatidylcholine biosynthesis[b]	Not yet elucidated.
BRUN	6–2388 (19555–20160); (18685–20160)	*LPD1* (dihydro- lipoamide dehydrogenase): involved in amino acid metabolism[b]	Not yet elucidated. Homologous genes in *S. pneumoniae* and *M. tuberculosis* encode virulence determinants (Smith *et al.*, 2002; Bryk *et al.*, 2002).
CA29	6–2454 (29747–30644); (29141–31129)	ORF6.6343: possible regulatory protein[b]	Not previously elucidated. Characterized further in this paper.
CH1	6–2502 (84666–85638); (83622–87782)	ORF6.7858: hypothetical membrane protein[b]	Not yet elucidated.

a. Contig and (positions), as assigned in the Stanford *Candida albicans* genome sequencing project database.
b. Putative gene function assigned by homology with genes of *S. cerevisiae*.

corresponded to the sizes deduced from the nucleotide sequences (Fig. 1).

Identification of C. albicans *genes encoding immunogenic antigens*

The sequences of the 10 ORFs were analysed by BLAST using the *C. albicans* and *Saccharomyces cerevisiae* genome databases (Stanford University Genome Technology Center and Institut Pasteur; Stanford University Genome Technology Center). All ORFs matched sequences within genes found in the *C. albicans* databases, enabling us to identify the complete gene sequences (Table 2). These included five previously identified genes that encode proteins of known function in *C. albicans* (*HWP1*, *CST20*, *CPP1*, *RBF1* and *PTH1*), three genes with names and functions assigned in the databases based on close identity with genes of *S. cerevisiae* (*CDC24*, *PCT1*, and *LPD1*), and two genes that have not yet been named or assigned functions (Stanford ORF 6.6343 and ORF 6.7858, identified from clones CA29 and CH1, respectively). ORF 6.6343 is 1986 bp in length and encodes a protein that has 30% identity with *S. cerevisiae* Not5p over a 401 amino acid segment of the N-terminus. The *C. albicans* gene corresponding to this ORF is further characterized below. ORF 6.7858 is 4161 bp in length and encodes a protein that has 42% identity with a hypothetical membrane protein encoded by *S. cerevisiae* YML059C.

The *C. albicans* genes encoding proteins of known or assigned function can be grouped into functional classes including regulation of morphogenesis (*CST20, CPP1* and *RBF1*) (Kohler and Fink, 1996; Csank *et al.*, 1997; Ishii *et al.*, 1997), adhesion to host cells (*HWP1*) (Staab *et al.*, 1999), nutrient uptake (*PTH1*), phospholipid biosynthesis and turnover (*PCT1*), and amino acid catabolism and transport (*LPD1* and *PTH1*). It is noteworthy that four genes (*CST20, CPP1, RBF1* and *HWP1*) encode previously identified determinants of candidal virulence (Kohler

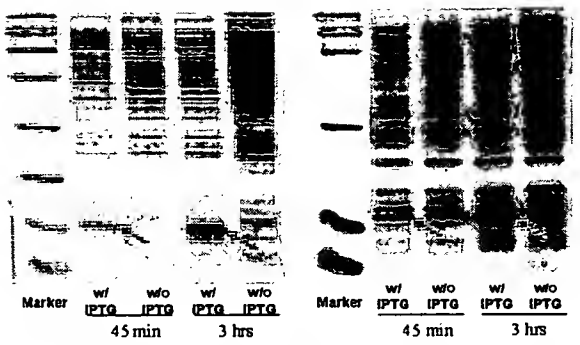


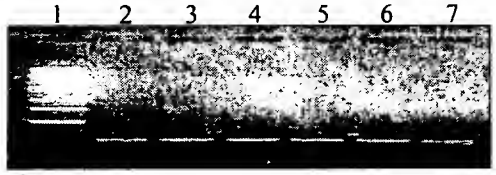
Fig. 1. Expression and immunoreactivity of a representative *C. albicans* antigen is verified by Commassie blue staining of an SDS-polyacrylamide gel (left) and Western analysis using adsorbed sera (right). The clone CA29, identified by screening, was incubated in LB-medium containing kanamycin (50 μg ml^{-1}) with and without IPTG (1 mM) for 45 min and 3 h. Following incubation, cell lysates were resolved by 15% SDS-PAGE. Commassie blue staining indicates that a unique polypeptide is induced in the presence of IPTG (arrows in the figure at left). Western analysis using pooled adsorbed sera obtained from HIV-infected patients with thrush (primary antibody) and peroxidase conjugated goat anti-human antibody (secondary antibody) demonstrates that the induced polypeptide is reactive with sera antibodies (arrows in the figure at right). The immunoreactive band is ~ 23 kDa, a size consistent with the size of the *C. albicans* genomic insert within CA29. The corresponding *C. albicans* gene was subsequently identified as *caNOT5*. Marker: protein standard. For Western analysis, the bands were visualized using ECL chemiluminescence.

and Fink, 1996; Ishii *et al.*, 1997; Guhad *et al.*, 1998; Staab *et al.*, 1999). In addition, *LPD1* encodes dihydrolipoamide dehydrogenase (DLDH), an enzyme that is related to a previously recognized bacterial virulence determinant. *Candida albicans* DLDH is 491 amino acids in length, and exhibits 40% and 38% homology with the DLDHs of *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* respectively. *Streptococcus pneumoniae* DLDH is necessary for virulence in murine models of pneumococcal sepsis and pneumonia (Smith *et al.*, 2002), and *M. tuberculosis* DLDH supports resistance to oxidative components of the immune response (Bryk *et al.*, 2002). The role of *LPD1* in the pathogenesis of candidiasis has not yet been investigated.

Confirmation of in vivo *expression of* C. albicans *genes within thrush pseudomembranes recovered from an HIV-infected patient*

We used reverse transcription-polymerase chain reaction (RT-PCR) to detect *C. albicans* transcripts within thrush samples collected from the oral cavity of an HIV-infected patient (Schaller *et al.*, 1998; Naglik *et al.*, 1999). Polymerase chain reactions were performed on cDNA templates using primers designed from the sequences of the 10 genes (Table 3). Specific primers were used to amplify a 526 basepair (bp) RT-PCR fragment of the *C. albicans* *EFB1* transcript, which lacked an intron of 365 bp. In the event of genomic DNA contamination, an 891 bp PCR fragment containing the intron would be amplified (Schaller *et al.*, 1998); this was not observed. The RT-PCR experiments proved that all 10 genes were unambiguously expressed during thrush (Table 3).

We also sought to determine whether the genes were expressed to a greater extent during thrush than under routine *in vitro* conditions. To accomplish this, we performed semiquantitative RT-PCR in parallel using cDNA templates prepared from *C. albicans* cells within the thrush sample and the same strain grown under *in vitro* conditions that favour growth as yeast (YPD liquid medium at 30°C) and hyphae (YPD medium supplemented with 5% calf serum at 37°C). This approach required that we initially establish an appropriate internal mRNA standard to control for potential differences in *C. albicans* concentrations and cell morphologies between the *in vivo* and *in vitro* samples. Previous studies suggested that *EFB1*, which is constitutively expressed, is suitable as such a control (Maneu *et al.*, 1996). To confirm this, we first demonstrated by RT-PCR that *EFB1* transcript concentrations were identical for equivalent numbers of *C. albicans* cells grown as yeast, hyphae, or varying mixtures of yeast and filamentous morphologies (Fig. 2). We next determined that the concentration of *C. albicans* in the thrush sample was 6×10^5 cells ml^{-1} by counting in a haemocytometer, and confirmed this by subsequent culture. The population



	Growth conditions	*In vivo*	RPMI1640+5%FBS at 37°C				
A	CFU ×100	90	93	89	97	88	84
B	% of yeast	60	100	90	40	10	0
C	% of pseudohyphae and hyphae	40	0	10	60	90	100

Fig. 2. EFB1 transcript concentrations determined by RT-PCR for equivalent numbers of *C. albicans* cells within a thrush sample (lane 2) or during growth as varying morphologies *in vitro* (lanes 3–7). RT-PCR was performed using *EFB1* specific primers on ~ 9000 CFU of the patient's *C. albicans* strain grown as varying percentages of yeast or pseudohyphae/hyphae *in vitro* (YPD medium at 30°C, lane 3; RPMI 1640 medium supplemented with 5% serum at 37°C for 15 min, 30 min, 60 min and 120 min; lanes 4–7, respectively). The relative intensities of the *EFB1* signals were quantified and compared to that of an *in vivo* sample containing an equivalent number of *C. albicans* cells (lane 2). The *C. albicans* CFUs used in the experiments are presented in row A, and the percentages of *C. albicans* cells growing as yeast or pseudohyphae/hyphae are presented in rows B and C respectively. Lane 1: molecular standard. Complete experimental details are provided in the *Experimental procedures* section. The *EFB1* signal intensities did not differ under the different conditions.

Table 3. Semi-quantitative RT-PCR comparing levels of *in vivo* and *in vitro* gene expression.[a]

IVIAT clone	*Candida albicans* gene	RT-PCR primers	RT-PCR signal (mean ± sd)/ thrush sample	RT-PCR signal (mean ± sd)/ yeast grown *in vitro*	RT-PCR signal (mean ± sd)/ hyphae grown *in vitro*	*P*-value[b]
(control)	*EFB1*	5′-ATTGAACGAATTCTTGGCTGAC-3′ 5′-CATCTTCTTCAACAGCAGCTTG-3′	106 ± 10	108 ± 10	109 ± 18	NS
CH7	*HWP1*	5′-ATGACTCCAGCTGGTTC-3′ 5′-TAGATCAAGAATGCAGC-3′	89 ± 14	Undetectable	16.6 ± 6	0.0007
CH3	*CST20*	5′-ATGTCTCATAATAATGGC-3′ 5′-GGTTAATTAGTTTCTTC-3′	96 ± 15	15 ± 12	16 ± 8	0.002
CH9	*CPP1*	5′-ATGTCTCATAATAATGGC-3′ 5′-TTAAAGGACTAGTGTACC-3′	92 ± 15	33 ± 15	12 ± 5	0.07
MA1	*RBF1*	5′-CATGTCAGAGATGCGCTCACT-3′ 5′-ATGACCAGGTTCATCGGGTT-3′	71 ± 1	26 ± 2	32 ± 5	0.0002
MA2	*PTH1*	5′-AAGGTACTAATGGGATGT-3′ 5′-CTTCAAGTGATATTGGTT-3′	30 ± 4	14 ± 7	10 ± 4	0.09
KHOI	*CDC24*	5′-CGGTGGTGATGAAGATGT-3′ 5′-CCAGGATCAAGTAGACTCGA-3′	111 ± 26	12 ± 3	14 ± 9	0.003
CLEM	*PCT1*	5′-AATAAAAAGAGAAAACAT-3′ 5′-TTACCCTTGTTCTTTTATAGG-3′	56 ± 9	9 ± 1	6 ± 4	0.01
BRUNO	*LPD1*	5′-TTGGCCCACAAAGCTGAA-3′ 5′-GTTGATTGGCTTATCAAA-3′	111 ± 25	15 ± 4	18 ± 10	0.02
CA29	ORF 6.6343	5′-ATGAATCCACCAAGGACG-3′ 5′-CCATTATCGGTAGAAGC-3′	123 ± 19	52 ± 12	16 ± 8	0.005
CH1	ORF 6.7858	5′-ATGTCTCATAATAATGGC-3′ 5′-GGTTTAATTAGTTTCTTC-3′	28 ± 20	1.4 ± 2.1	Undetectable	0.08

a. Reactions were performed under the following conditions: 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C, preceded by denaturation for 15 min at 94°C and followed by a final extension cycle for 10 min at 72°C. Reactions were carried out for 20, 25, 30, 35, and 40 cycles in order to verify that expression ratios remained constant until maximal amplification intensity was achieved. Before maximal amplification, expression ratios were consistent for differing numbers of cycles for each of the genes. In this table, the data is for amplification of 35 cycles for all genes except *LPD1*, for which amplification of 30 cycles is presented.
b. *P*-value determined by three-way ANOVA.

consisted of approximately 60% yeast and 40% filamentous morphologies. Finally, we demonstrated that *EFB1* transcript concentrations were equivalent within our *in vivo* and *in vitro* samples if we started with the same number of *C. albicans* cells (Fig. 2).

Having confirmed the validity of our internal control, we then performed RT-PCR over a range of amplification cycles for *EFB1* and the 10 genes identified during screening. In three independent experiments, the RT-PCR signals for the 10 genes within the thrush sample were consistently higher than the signals for the corresponding genes under *in vitro* conditions favouring yeast cells (two-fold to 89-fold) or hyphae (threefold to 28-fold) (Table 3; Fig. 3). Six genes were expressed to an equivalent degree *in vitro* by yeast and hyphae, two genes were expressed to a greater degree by yeast than hyphae (*CPP1* and ORF 6.6343), and one gene was expressed solely by hyphae (*HWP1*).

In addition to verifying that the 10 genes were expressed *in vivo*, we directly visualized two representative antigens in association with *C. albicans* cells within thrush samples recovered from our patient. We purified CA29p (i.e. the protein expressed by clone CA29) and Pth1p, and raised primary antibodies in mice (Ou *et al.*, 1993). Using these antibodies as probes, CA29p was localized to the walls of *C. albicans* yeast cells within the thrush samples (Fig. 4A), and Pth1p was localized in a beading pattern to the walls of hyphal elements (Fig. 4C). Neither CA29p nor Pth1p were detected in association with patient's bacterial oral flora or epithelial cells, nor did antibodies obtained from mice that had not been inoculated with the antigens react with candidal cells. In each experiment, negative control slides were probed with secondary antibody but not with anti-CA29p or anti-Pthp antibodies (Figs 4B and D).

Characterization of the C. albicans *gene corresponding to ORF 6.6343*

We further characterized the gene encoding CA29 (ORF 6.6343). Analysis of the putative amino acid sequence revealed two domains consistent with possible regulatory elements (amino acid positions 1–40 and 291–662), and demonstrated identity with the N-terminus of *S. cerevisiae* Not5p. In *S. cerevisiae,* Not5p is a component of the CCR4-NOT transcriptional complex, which regulates a wide range of processes including filamentation and maintenance of cell wall integrity (Mosch and Fink, 1997; Liu *et al.*, 1998; Oberholzer and Collart, 1998). Starting with CAI4, a *ura3- C. albicans* strain that was derived from the

Fig. 3. Relative expression of EFB1 (lanes 1–3), HWP1 (lanes 4–6), and RBF1 (lanes 7–9) determined by RT-PCR under *in vitro* and *in vivo* conditions. The RT-PCR results after 35 cycles are represented for *C. albicans* grown as yeast *in vitro* (YPD at 30°C; lanes 1, 4, 7), hyphae *in vitro* (YPD supplemented with 5% fetal calf serum at 37°C; lanes 2, 5, 8), and within an *in vivo* thrush sample (lanes 3, 6, 9). *EFB1* transcript concentrations were similar under all three conditions (lanes 1–3). *HWP1* transcript concentrations were significantly greater *in vivo* (lane 6) than under *in vitro* hyphal conditions (lane 5); transcripts could not be detected under *in vitro* yeast conditions (lane 4). *RBF1* transcript concentrations were higher *in vivo* (lane 9) than under either *in vitro* condition (lanes 7, 8). Quantification of band intensities is provided in Table 3. M: DNA marker. Complete experimental details are provided in the *Experimental procedures* section.

clinical strain SC5314, we constructed isogenic strains in which one or both alleles of the CA29 gene were disrupted by the URA3 blaster protocol (Table 4; Fig. 5): CA29-1 (heterozygous mutant) and CA29-3 (null mutant). Both disrupted strains demonstrated the same overall growth rate as the URA3 + parent strains SC5314 and CAF2-1 in YPD liquid media at 37°C. The null mutant strain (CA29-3), however, exhibited complete loss of hyphal formation at the periphery of colonies grown on hyphal-inducing solid media (YPD supplemented with 5% calf serum, M199, Spider and modified Lee's media) (Fig. 6). In hyphal-inducing liquid media (YPD supplemented with 5% calf serum; RPMI 1640), CAF2-1 developed germ tubes and true hyphae within 30 min, whereas the null mutant either remained as yeast cells or could form only truncated germ tubes; after two hours of incubation, CAF2-1 exhibited dense mats of extended hyphae, whereas the null mutant continued to exhibit truncated germ tubes but no true hyphae (Fig. 7). The heterozygous mutant strain (CA29-1) exhibited intermediate phenotypes in hyphal-inducing liquid media (Fig. 7). On hyphal-inducing solid

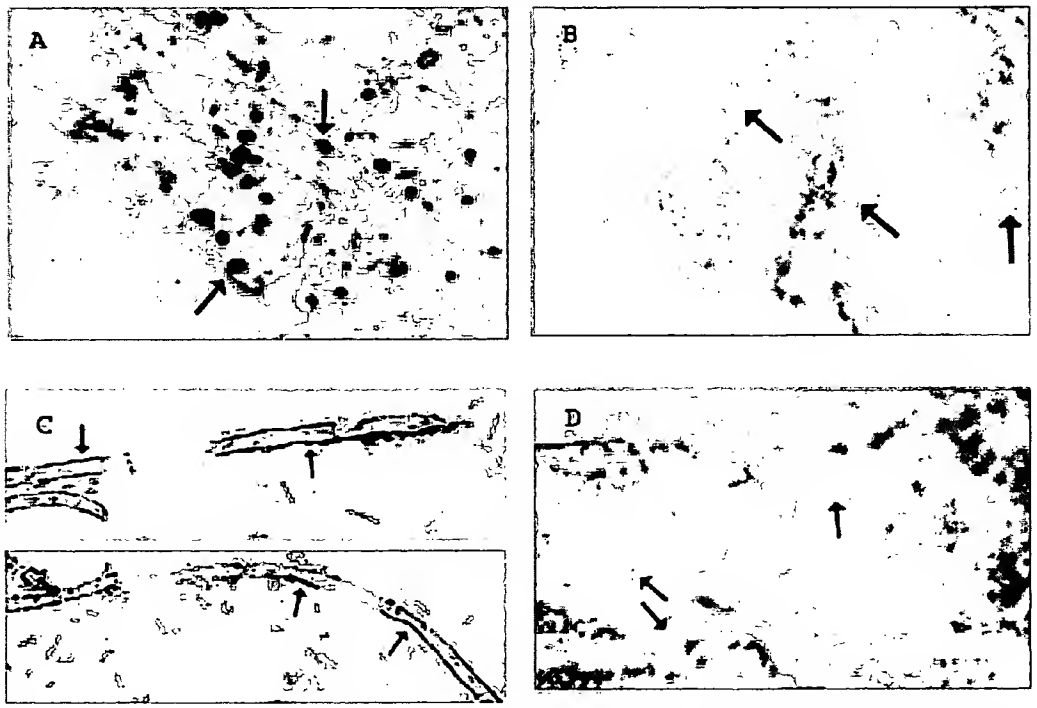


Fig. 4. CA29p and Pth1p are detected in association with *C. albicans* cells during active thrush. Polyclonal antibodies (anti-CA29p, anti-Pth1p) were raised in mice against purified antigens. A horseradish peroxidase procedure for light microscopy was used to visualize pseudomembranes recovered from the oral cavity of an HIV-infected patient with thrush, following probing with anti-CA29p or anti-Pth1p and biotinylated horse anti-mouse IgG. Multiple slides were prepared from a single tissue sample and were probed in parallel. In each of the slides, epithelial cells and bacterial flora appear as a light brown background.
A. CA29p stains dark brown and is associated with spherical yeast and budding yeast cells (arrows), which are adherent to epithelial cells (200×).
B. A negative control slide, processed identically except for the omission of the anti-CA29p primary antibody. *C. albicans* appears as yeast cells that do not stain (arrows).
C. Pth1p is detected along the parallel walls of hyphal elements in an irregular, beading fashion (arrows) (600×).
D. A negative control slide for which anti-Pth1p primary antibody was omitted shows non-staining hyphal elements (200×).

Table 4. *Candida albicans* strains created in this study.

Strain	Genotype (reference)
SC5314	Clinical isolate (Gillum *et al.*, 1984)
CAF2-1	*Δura3::imm434/URA3* (Fonzi and Irwin, 1993)
CAI4	*Δura3::imm434/Δura3::imm434* (Fonzi and Irwin, 1993)
CA29-1,CA29A	*CA29/Δca29::hisG-URA3-hisG Δura3::imm434/Δura3::imm434*
CA29-2,CA29B	*CA29/Δca29::hisG Δura3::imm434/Δura3::imm434*
CA29-3,CA29C	*Δca29::hisG-URA3-hisG/Δca29::hisG Δura3::imm434/Δura3::imm434*
CA29-4,CA29D	*Δca29::hisG/Δca29::hisG Δura3::imm434/Δura3::imm434*
CA29-5,CA29E	*Δca29::hisG/CA29::URA3-hisG Δura3::imm434/Δura3::imm434*

CAI4 was the parent strain for all disrupted strains created in this study. All strains are ultimately derived from SC5314.

media, CA29-1 more closely resembled the parent strains SC5314 and CAF-2 (Fig. 6). Reintroduction of a copy of the gene at a disrupted locus in the null mutant (creating strain CA29-5) rescued the phenotypes (Figs 6 and 7). In addition to defects in hyphal formation, the null mutant was unable to grow on YPD media containing 0.02% SDS, consistent with defects in cell wall integrity (Liu *et al.*, 1998). Unlike the phenotypes observed in *S. cerevisiae NOT5* null mutant strains, the CA29 null mutant was not sensitive to caffeine, $MgCl_2$ or calcofluor white, and was sensitive to heat shock at 50°C.

In order to verify the validity of the phenotypes we observed, we re-performed the disruptions and re-insertion of the CA29 gene, creating strains CA29A-CA29E (corresponding to CA29-1-CA29-5) (Table 4). Results of the experiments performed with the second set of strains were identical. Based upon sequence homology and the effects of gene disruption therefore we propose that CA29, the *C. albicans* gene corresponding to ORF 6.6343, be named *CaNOT5*.

Determination of the roles of CaNOT5 in the adherence *to buccal epithelial cells and the pathogenesis of murine candidiasis*

To prove that our screening strategy identifies previously unrecognized candidal virulence determinants, we first assessed the effects of *CaNOT5* disruption in a cell model of oral candidiasis (Tsang and Samaranayake, 1999). Freshly harvested human buccal epithelial cells (BECs) were infected with *C. albicans* strains at 37°C for one hour. The adherence of the parent (CAF2-1) and the heterozygous mutant (CA29-1) strains to BECs were significantly higher than that of the null mutant (CA29-3) (69.5% ± 0.7, 58.5% ± 2.1, 39% ± 1.4, respectively; $P = 0.005$, 2-way ANOVA).

We further assessed the effects of *CaNOT5* disruption in a murine model of disseminated candidiasis. Groups of 12 ICR mice were infected intravenously via the lateral tail vein with 10^6 CFU/mouse. All mice infected with the parent strain (CAF2-1) died by day 6, and all mice infected with the heterozygous mutant (CA29-1) or reinsertion strains (CA29-5) died by day 10 (Fig. 8). In contrast, the first death among mice infected with the *CaNOT5* null mutant strain (CA29-3) was not observed until day 9. Indeed, 42% of mice infected with the null mutant strain were still alive after 30 days. The mean survival times for mice infected with the parent, heterozygous mutant, and reinsertion strains were 4.3 ± 1.0 days, 8.6 ± 0.7 days and 9.5 ± 0.5 days, respectively, whereas the mean survival for the null mutant was 20.8 ± 9.4 days. Newman Keuls multiple contrast analysis revealed that mice infected with the null mutant strain survived significantly longer than mice infected with the heterozygous mutant ($P = 0.03$) and the parent strains ($P < 0.0001$). There was no significant difference in the survival time between mice infected with the heterozygous mutant and reinsertion strains. The murine studies were repeated with strains CA29A-CA29E, with reproducible results (data not shown).

Discussion

The major strength of this study is that it describes a novel approach to an important problem in the study of candidal pathogenesis: the identification of *C. albicans* genes expressed during human infections. To date, large-scale screening using existing technologies to identify *in vivo* expressed *C. albicans* genes has not been practical. In this study, we used an antibody-based screening method to identify 10 candidal genes that are induced in HIV-infected patients with active thrush. These genes are involved in a wide range of biologic processes likely to be crucial for optimal survival within the human host, including regulation of morphogenesis, adhesion to host cells via cell surface proteins, nutrient uptake, phospholipid biosynthesis and turnover, and amino acid catabolism and transport. As such, the genes might be thought of as encoding potential virulence determinants that optimize *C. albicans'* ability to colonize or invade host tissues.

Indeed, our results confirm that selected *in vivo* induced genes encode virulence determinants. Four genes were previously known to be involved in virulence: targeted disruptions of *HWP1*, *CST20*, *CPP1* and *RBF1* attenuated virulence in murine models of disseminated candidiasis

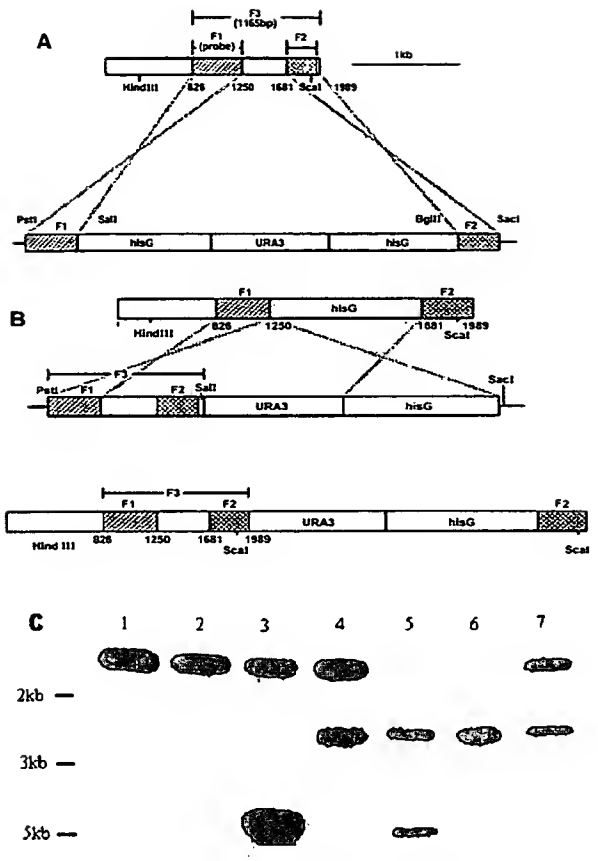


Fig. 5. Targeted disruption and re-insertion of *C. albicans* ORF 6.6343 in CAI4.

A. Schematic diagram of the disruption protocol. The top figure represents an allele of ORF 6.6343 in strain CAI4. The bottom figure shows the disruption cassette created in pMB7, in which *hisG-URA3-hisG* is flanked by ORF 6.6343 fragments F1 (nucleotides 826–1250) and F2 (nucleotides 1681–1978). The cassette was released by digestion with *PstI* and *SacI* and transformed into strain CAI4 by electroporation. Recombination is indicated by the dashed lines.

B. Reinsertion protocol. The top figure shows a disrupted ORF 6.6343 allele, as in strain CA29-4. The figure below it shows the construction of the gene re-insertion cassette. pMB-7 was first digested with *Xba*I to remove a copy of *hisG*, creating pMB7-1. ORF 6.6343 fragment F3 (nucleotides 826–1990, which includes the 3′end of the gene) was next subcloned proximally to the *URA3* region. The resulting cassette was released from the plasmid by digestion with *PstI* and *SacI*, and transformed into strain CA29-4. Recombination is indicated by the dashed lines. The bottom figure provides a schematic representation of the revertant allele, as in strain CA29-5. As shown, a complete copy of ORF 6.6343 is reconstituted.

C. Southern blot verifying each step in the disruption. Genomic DNA was prepared for strains SC5314, CAI4, CA29-1, CA29-2, CA29-3, CA29-4 and CA29-5 (lanes 1–7, respectively), digested with *Hin*dIII and *ScaI*, separated by electrophoresis on agar gels and transferred to a nylon membrane. The reactive bands are hybridized with radiolabelled F1 fragment as a probe (425 bp). The positions of the molecular weight marker are indicated on the left.

(Kohler and Fink, 1996; Ishii *et al.*, 1997; Guhad *et al.*, 1998; Staab *et al.*, 1999). In addition, another gene, *LPD1*, has not been previously studied for a possible role in candidal pathogenesis but might prove to be important because it encodes a protein that is homologous to a virulence determinant for *S. pneumoniae* and *M. tuberculosis* (Bryk *et al.*, 2002; Smith *et al.*, 2002). Most importantly, we showed that an *in vivo* induced gene identified in this study, which we call *CaNOT5* based on homology with *NOT5* of the baker's yeast *S. cerevisiae*, encodes a previously unrecognized protein involved in the pathogenic process. Targeted disruption of *CaNOT5* decreased the adherence of *C. albicans* cells to human buccal epithelial cells, as well as significantly attenuated mortality in mice with disseminated candidiasis.

It is noteworthy that the five genes encoding virulence determinants during thrush are involved in different aspects of *C. albicans* morphogenesis. Two genes (*CPP1* and *RBF1*) are negative regulators of filamentous growth, and a third gene (*CST20*) is a positive regulator (Kohler and Fink, 1996; Csank *et al.*, 1997; Ishii *et al.*, 1997). Two other genes, *HWP1* and *CaNOT5*, encode proteins that are required for normal hyphal formation (Staab *et al.*, 1999; this study). Furthermore, it is possible that other *in vivo* induced genes identified in this study are also associated with morphogenesis. *CDC24*, for example, encodes a protein that has 34% identity with *S. cerevisiae* Cdc24p, which is involved in apical bud formation and pseudohyphal growth. Taken together, our findings support the theory that candidal pathogenesis within human depends, at least in part, upon the organism's ability to exist in both yeast and filamentous morphologies, as well as by the ability to switch between morphologies in response to changing local host conditions (Calderone *et al.*, 2001).

We have demonstrated that both intracellular and cell surface candidal antigens are immunogenic in humans. The identification of immunogenic intracellular antigens is not surprising as it confirms the results of several earlier studies. Indeed, a diverse range of intracellular candidal proteins have been shown to be highly immunogenic, including glycolytic enzymes (Swoboda *et al.*, 1993; Martinez *et al.*, 1998; Pitarch *et al.*, 1999; Pardo *et al.*, 2000), metabolic enzymes (Pardo *et al.*, 2000), and heat shock proteins (Lopez-Ribot *et al.*, 1996; Eroles *et al.*, 1997; Pitarch *et al.*, 2001). There are two hypothesized explanations for this finding. First, several proteins predicted to be intracellular based on sequence motifs or molecular functions have been shown to localize to the surface of *C. albicans* cells. These include enolase (Eroles *et al.*, 1997), phosphoglycerate kinase (Alloush *et al.*, 1997), alcohol dehydrogenase (Pendrak and Klotz, 1995), glyceraldehyde 3-phosphate dehydrogenase (Gil-Navarro *et al.*, 1997) and Hsp70 proteins (Lopez-Ribot *et al.*, 1996; Eroles *et al.*, 1997). Dihydrolipoamide dehydrogenase (DLDH), the enzyme encoded by the *in vivo*-induced gene *LPD1*, might be similar to these proteins. *Neisseria meningitidis* DLDH, for example, is highly immunogenic and

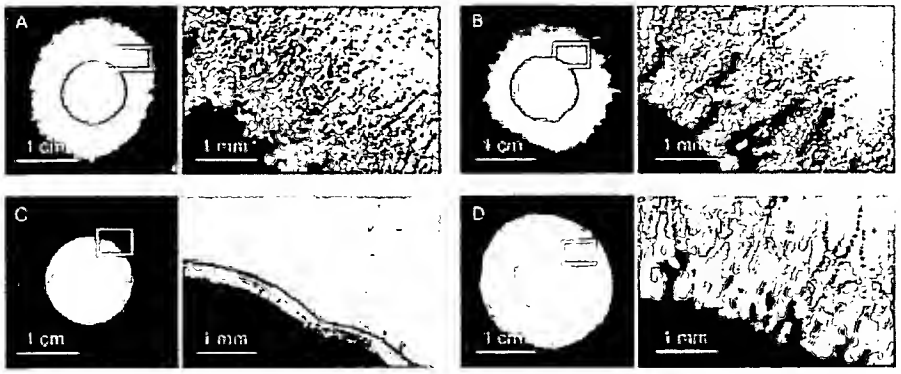


Fig. 6. Effects of CA29 gene disruption on hyphal formation by *C. albicans* in solid medium. Colonies of isogenic *C. albicans* strains were photographed after incubation at 37°C for 5 days on solid Spider medium.
A. Parent strain (CAF2-1).
B. CA29 heterozygous mutant strain (CA29-1).
C. CA29 null mutant strain (CA29-3).
D. CA29 reinsertion strain (CA29-5).
For each strain, the colony edge indicated in the left-hand photo is magnified (×10) in the right-hand photo.

can be localized to the bacterial cell surface (Li de la Sierra *et al.*, 1997; Exposito Raya *et al.*, 1999). As an alternative explanation for the immunogenicity of certain intracellular proteins, it is possible that antibodies are mounted against proteins that are released from *C. albicans* cells after damage or lysis caused by the human immune system. This scenario has been suggested for the elevated antibody levels against the mitochondrial enzyme aconitate hydratase among patients with disseminated candidiasis (Pardo *et al.*, 2000).

A number of immunogenic antigens have previously been identified by using human or animal sera to screen *C. albicans* cDNA libraries and two-dimensional (2D) protein gels (Swoboda *et al.*, 1993; Alloush *et al.*, 1997; Gil-Navarro *et al.*, 1997; Pitarch *et al.*, 1999; 2000). The fact that we found immunogenic antigens not identified by these studies might be explained by differences in the methodologies. The cDNA libraries in the earlier studies were created from *C. albicans* cells grown under hyphal-inducing conditions *in vitro*, and were probed with either sera recovered from humans with candidiasis (Swoboda *et al.*, 1993; Gil-Navarro *et al.*, 1997) or from rabbits immunized with candidal cell wall proteins (Alloush *et al.*, 1997). In the latter case, the pool of antibodies was likely to be more limited than ours, because it included only those directed against the specific proteins isolated by the investigators. Furthermore, whereas our approach will eventually allow screening of virtually the entire *C. albicans* genome, the cDNA libraries were limited to those mRNA transcripts present in sufficient concentrations under specific *in vitro* conditions. Genes expressed exclusively within the human host or expressed minimally *in vitro* might be excluded. This could be particularly relevant for genes whose transcripts are likely to be present in low copy numbers, such as those encoding transcription factors, regulatory proteins, MAP kinases and MAP kinase phosphatases. Protein separation by 2D gel electrophoresis has similar potential limitations, as only the most abundant proteins will be resolved (Pitarch *et al.*, 1999). In the

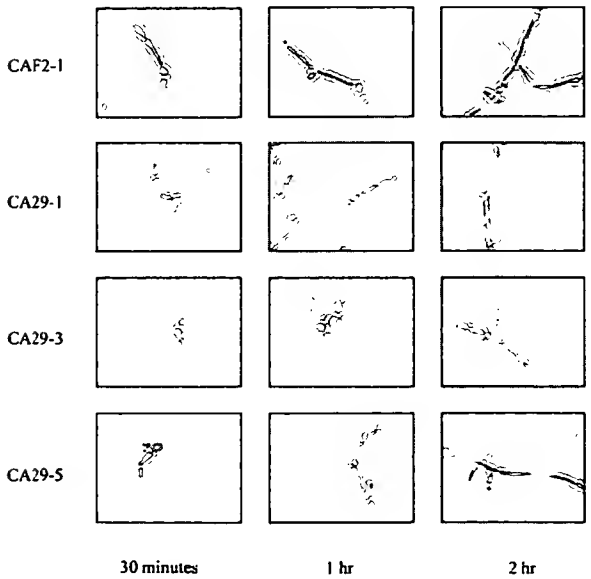


Fig. 7. Effects of CA29 gene disruption on hyphal formation by *C. albicans* in liquid medium. Colonies of *C. albicans* isogenic strains were photographed after incubation at 37°C for 0.5, 1 and 2 h in YPD medium supplemented with 5% fetal calf serum.

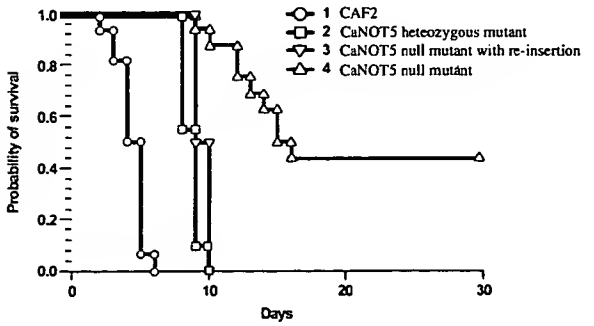


Fig. 8. Effects of *CaNOT5* disruption on survival of ICR mice infected intravenously with 10^6 CFU of *C. albicans*. Mice were infected with a *C. albicans* parent strain (#1, CAF2-1), a *CaNOT5* heterozygous mutant strain (#2, CA29-1), a *CaNOT5* null mutant strain (#4, CA29-3), or a *CaNOT5* reinsertion strain (#3, CA29-5).

case of the *S. cerevisiae*, for example, analysis of medium and low abundance proteins is limited even with optimized techniques, as proteins from about one-half of all genes are not represented (Gygi *et al.*, 2000).

In addition to its technical feasibility, our screening strategy has the advantage over existing *in vivo* expression technologies of detecting gene expression within human hosts, rather than relying upon extrapolations from animal models. The promising findings from a partial screen of the *C. albicans* genome in this study suggest that screening the complete genome might identify a number of genes that contribute to the pathogenic process. Based upon the average size of genomic inserts in our library, a theoretical minimum of 400 000 clones would be necessary to assure that the library contained each ORF at least once (Sambrook and Russell, 2001). We must acknowledge, however, that even with screening the complete genome, our method will not identify every *C. albicans* gene expressed within the human host or every virulence determinant. For example, we will not identify genes encoding proteins that are non-immunogenic, genes that cannot be expressed by *E. coli,* or genes whose products are toxic to the bacteria. In addition, genes that are well expressed both *in vitro* and *in vivo* should not be identified. By the design, of course, non-protein factors cannot be detected, even though some of these are important to the pathogenic process. Finally, our method does not account for possible post-translational modifications of proteins that might occur within *C. albicans* cells.

Although we have demonstrated that all 10 genes are expressed within the oral cavity during active thrush, we cannot exclude that some or all of the genes might also be expressed at other tissue sites during invasive candidiasis or routine colonization. To assess this, we are currently collecting serum from patients with diverse types of candidal infections, as well as from uninfected controls, for which antibody concentrations against each of the *in vivo* induced antigens will be determined. We also plan to simultaneously screen the expression library with diverse pools of sera in parallel, with the goal of identifying genes that are expressed uniquely during specific infections or at particular tissue sites. Hopefully, future studies such as these will advance our understanding of candidal pathogenesis by identifying genes that are associated with the infectious process, as well as genes contributing to survival as a commensal organism.

Experimental procedures

Strains and growth conditions

Candida albicans library and plasmid constructs were propagated in *Escherichia coli* DH5α grown in Luria–Bertani (LB) medium with the appropriate antibiotic selection. Overexpression experiments were performed using *E. coli* BL21(DE3) grown in LB medium with 50 μg ml^{-1} kanamycin and 1 mM IPTG.

Candida albicans strains used to construct the genomic library were 24 clinical isolates obtained from unique patients with thrush. *Candida albicans* SC5314 was the parent strain for subsequent gene disruption and reinsertion experiments (Fonzi and Irwin, 1993). All strains were routinely grown in YPD medium (1% yeast extract, 1% bactopeptone, 2% a[D]-glucose) at 30°C, unless otherwise noted. To induce hyphal formation by *C. albicans* strains, both solid and liquid media were tested. The hyphal-inducing solid agar media used were: Spider medium (Gimeno *et al.*, 1992.), Medium 199 (M-199) (Gibco-BRL, adjusted to pH 7.5) (Ramon *et al.*, 1999), modified Lee's (Lee *et al.*, 1975), and YPD medium supplemented with 5% fetal calf serum. The hyphal-inducing liquid media used were YPD supplemented with 5% fetal calf serum and RPMI 1640.

Genomic expression library construction

Genomic DNA was purified from 24 clinical *C. albicans* isolates. Equivalent amounts of purified genomic DNA from the isolates were pooled and partially digested with *Sau3A*1 to yield 0.5–2 kb fragments. The gel-purified fragments were ligated into pET30abc inducible expression vectors (Novagen) that had been digested with *BamH1* and dephosphorylated with calf intestinal phosphatase, and then transformed into *E. coli* DH5α. The library was spread onto LB agar containing 50 μg ml^{-1} kanamycin. After overnight incubation at 37°C, the plates were scraped to collect the transformed cells. Plasmid DNA obtained from these cells was used to transform *E. coli* BL21(DE3). Transformed cells were collected and aliquoted to vials containing LB media with 15% glycerol for storage at −70°C.

Adsorption of human sera (Brady et al.,*1998)*

A clinical *C. albicans* strain (*Ca* 172) was grown in YPD broth at 37°C. A wet-mount preparation of the culture revealed a mixed population of cell morphologies: the majority of cells were single or budding yeasts, with pseudohyphal and hyphal elements also observed. Each adsorption consisted of an overnight incubation of the pooled sera with approximately 10^8 cells in 0.1 ml phosphate-buffered saline (PBS) containing 0.02% sodium azide with mild agitation at 4°C. Subsequently, the sera were further adsorbed by exposure to a nitrocellulose membrane coated with French-press cell extracts and heat denatured cell extracts of *in vitro* grown *C. albicans*. To measure the efficacy of the absorption steps, *Ca* 172 was grown under the conditions used to adsorb the sera and French-pressed cell extracts were immobilized in microtitre wells. A modified ELISA procedure was used to test serial dilutions of pooled sera samples taken after each round of adsorption (Ebersole *et al.*, 1980).

Screening a C. albicans *genomic expression library with adsorbed sera*

The genomic expression library was grown on LB medium containing kanamycin (50 mg ml^{-1}) for 12–14 h at 37°C to

generate plates containing approximately 200–500 colonies. Each plate was replicated using sterile velvet onto a LB plate containing kanamycin and IPTG (1 mM) and incubated for 5 h at 37°C to induce expression of cloned open reading frames (ORFs). The colonies were exposed to chloroform vapours for 20 min, then overlaid with nitrocellulose membranes. The membranes were incubated in PBS, pH 7.2, containing 0.5% Tween-20 (PBS-Tween) and 5% non-fat skim-milk. They were reacted with the pooled adsorbed sera at a 1:5000 dilution in PBS-Tween for 1 h, then with peroxidase-conjugated goat anti-human immunoglobulin (Cappel/ICN) at a 1:5000 dilution for 1 h. Reactive clones were detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia) and exposure to Hyperfilm (Amersham Pharmacia). In preliminary experiments, we determined that it was unnecessary to adsorb the sera against *E. coli* BL21(DE3); although there was some reactivity between host strain proteins and sera, this resulted in a relatively low background compared to signals produced by immunogenic *C. albicans* antigens.

To confirm the immunoreactivity of the clones identified above, several colonies from the master plate in the apparent vicinity of the signal were streaked for single colony isolation onto LB plates. After overnight incubation, three single colonies isolated from each original colony were resuspended in fresh liquid LB medium containing kanamycin. Aliquots were blotted onto solid LB medium containing kanamycin and IPTG. Following the induction of recombinant protein expression, the blotted cells were lysed and re-screened using the same procedures as in the first round of screening. Two negative controls were included on each plate: pET30b/BL21(DE3) with no cloned insert, and a random clone that contained an insert but was non-reactive with sera. After preliminary screening identified *C. albicans* Hwp1p as an immunogenic antigen, the clone expressing this antigen was included on each plate as a positive control.

Western analysis

Individual clones that were reactive with pooled adsorbed sera were optimized for protein production and analysed by Coomassie brilliant blue staining and Western analysis of SDS-PAGE gels. The reactive protein bands were identified using adsorbed pooled patient sera as primary antibodies, followed by incubation with peroxidase conjugated goat anti-human antibody (ICN) as secondary antibodies. The bands were visualized using the ECL chemiluminescent method (Amersham).

Thrush pseudomembrane samples

Thrush samples were collected from the oral cavity of an HIV-infected patient using a sterile wooden blade, dispensed into a 1.2 ml LB medium, and immediately flash frozen and stored at −70°C in LB media with 15% glycerol in order to preserve RNA integrity. At the same time, additional samples from the same oral cavity sites were collected using a sterile brush swab, and were smeared immediately onto gelatin-coated slides. Multiple slides could be prepared from each sample. The slides were fixed at the bedside with 2% paraformaldehyde-lysine-periodate then stored at −20°C for use in the immunohistochemistry experiments. The presence of *C. albicans* in the thrush samples was confirmed by KOH smear, and by culture onto Sabouraud dextrose agar (SDA). All samples contained pure growths of *C. albicans*.

Reverse transcription-polymerase chain reaction (RT-PCR)

At the time of the RT-PCR experiments, total RNA was extracted from thawed samples and cDNA made by established methods (Schaller *et al.*, 1998; Naglik *et al.*, 1999). Polymerase chain reactions were performed on cDNA templates using primers designed from the sequences of the 10 genes. Polymerase chain reaction was performed using the following protocol: 1 minute at 94°C, 1 min at 50°C, and 1 min at 72°C, preceded by denaturation for 5 min at 94°C and followed by a final extension cycle for 10 min at 72°C. Reactions were carried out at 20, 25, 30, 35, and 40 cycles in order to verify that expression ratios remained constant until maximal amplification intensity was achieved. We controlled for the absence of genomic DNA contamination by including the constitutively expressed housekeeping gene *EFB1* (elongation factor 1β) as an internal mRNA control (Schaller *et al.*, 1998; Naglik *et al.*, 1999; Ripeau *et al.*, 2002). Polymerase chain reaction products were sequenced to verify that the desired *C. albicans* genes were amplified.

To compare relative levels of transcript concentrations in *in vivo* and *in vitro* samples by semiquantitative RT-PCR, we first established that *EFB1* was an appropriate internal mRNA standard. After thorough mixing to create a uniform sample, 1.2 ml of thawed sample was pipeted in 0.6 ml aliquots to each of two Eppendorf microcentrifuge tubes. The sample in the first tube was used for total RNA extraction and synthesis of cDNA, as above. The sample in the second tube was serially diluted for colony count by haemocytometer, and subsequent verification by plating on SDA media. Next, we extracted total RNA and made cDNA from an equal quantity of the patient's *C. albicans* strain grown under the *in vitro* conditions that favoured growth as yeast (YPD medium at 30°C) or hyphae (YPD medium supplemented with 5% fetal calf serum at 37°C; RPMI 1640 medium supplemented with 5% fetal calf serum). Polymerase chain reactions using the *EFB1* primers were performed on the same number of *C. albicans* cells using *in vivo* and *in vitro* samples over cycling lengths of 20, 25, 30, 35, and 40. Measurements of amplified band intensities were made with the ABI PRISM 7700 SDS instrument (Applied Biosystems) based on ethidium bromide staining, and concentrations determined using a standard curve derived from serial dilutions of 0.5 µg of a standard 1.0 kb DNA ladder (New England Biolabs). Transcript concentrations for each gene were normalized based on comparison to *EFB1*. All experiments were performed in triplicate, and the average and standard deviation of concentration of each band calculated. Three-way ANOVA was used to determine the statistical differences in concentrations between the *in vivo* and *in vitro* samples; *P*-values ≤ 0.05 were considered statistically significant.

Immunohistochemistry (Verlander et al., *1996)*

The gelatin-coated slides containing thrush pseudomem-

branes were fixed with ethanol and washed with PBS. Antigen retrieval was undertaken by heating the slides in a solution of 0.1 M citric acid, 0.1 M sodium citrate, pH 6.0, followed by cooling to room temperature. Each of the *in vivo* induced antigens was purified by nickel chelation chromatography using the His-bind Kit (Novagen). The primary antibodies were raised in mice against the purified *in vivo* induced antigens by contract with the University of Florida Core Facility using their standard protocols (Ou *et al.*, 1993); these antibodies were diluted to 1:1000 in PBS. The secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:250 in PBS. Following incubation with the primary and secondary antibodies, slides were washed in PBS and treated with avidin-biotin complex reagent (Vector ABC Kit, Vector Laboratories). Following another PBS wash, slides were exposed to diaminobenzidine in imidazole buffer with 0.3% H_2O_2, washed in distilled water, and counterstained with hematoxylin. Slides were viewed by light microscopy. Negative control slides were processed in parallel with the slides included in the study. Negative controls included fixed thrush pseudomembranes but no primary antibody, thrush pseudomembranes probed with primary antibody recovered from mice not immunized with purified antigen, slides of uninfected buccal mucosa collected from the same patient, and slides of uninfected buccal mucosa recovered from other HIV-infected patients who did not have active thrush.

Targeted disruption and re-insertion of C. albicans *ORF 6.6343 (Fig. 5)*

Sequential disruptions of both alleles of ORF 6.6343 (*CaNOT5*) and subsequent chromosomal re-insertion of an allele were performed using standard protocols (Fonzi and Irwin, 1993; Calera *et al.*, 1999). A proximal fragment of ORF 6.6343 from positions 826–1250 was amplified by PCR using the primers CA29F1-FOR (5′-AACCAGCTGCAGAATCCACC AAGGACGTCTT-3′) and CA29F1-REV (5′-CCAAGTGTCGA CGGCGGTAAAACTTTAACT-3′), which contained the introduced *Pst*I and *Sal*I restriction sites respectively (underlined). A distal gene fragment from positions 1681–1918 was amplified by PCR using the primers CA29F2-FOR (5′-AACCTC AGATGTTCGAAATACAATGCAGCC-3′) and CA29F2-REV (5′-TTCACCGAGCTCTCTGTCTGTTTTCAGTAG-3′), which contained the introduced *Bgl*II and *Sac*I restriction sites (underlined). Following amplication, the fragments were digested with the appropriate restriction enzymes and ligated sequentially into the plasmid pMB-7, flanking the *hisG-URA3-hisG* disruption cassette. The resulting plasmid was digested with *Pst*I and *Sac*I and transformed into *C. albicans* strain CAI4 by electroporation. Ura + transformants were selected on SD plates lacking uracil. After confirmation of disruption by Southern analysis, a Ura + transformant (strain CA29-1) was screened for segregants on 5-fluoroortic acid (5-FOA) plates. This *ura*-strain (CA29-2) was transformed with the same disruption cassette to disrupt the second copy of ORF 6.6343 (creating CA29-3). For Southern analysis of genotypes, genomic DNA from the transformants (1–2 mg) was digested with *Hin*dIII and *Sca*I. Digested DNA was hybridized with a ^{32}P-labelled fragment of ORF 6.6343 (position 826–1918) obtained by PCR using primers CA29F1-FOR and CA29F2-REV.

To construct CA29-5, a *C. albicans* strain with one ORF 6.6343 allele reconstituted, we first constructed a modified pMB7 (pMB7-1) in which one copy of *hisG* was eliminated by digestion with *Xba*I followed by religation. Then, ORF 6.6343 was amplified from position 826–1990 by PCR using the primers CA29F1-FOR and CA29R1 (5′-ATCGGTGTC GACTCATTGGAAAATCTGTCTG-3′; *Sal*I site underlined). This fragment was subcloned into pMB7-1 following *Pst*I and *Sal*I digestion. The reinsertion cassette was released by digestion with *Pst*I and *Sac*I and used to transform CA29-4, a *ura*-ORF 6.6343 disruption strain created from CA29-3 by selection on 5-FOA. Reinsertion was confirmed by Southern analysis.

Adherence of C. albicans *to human buccal epithelial cells (BECs) (Tsang and Samaranayake, 1999)*

Buccal epithelial cells were collected from three investigators by gently scraping the cheek mucosa with a cotton swab and dispensed into 10 ml PBS. The pooled BECs were then washed four times with PBS, and counted using a haemocytometer. A final concentration of 1×10^5 epithelial cells ml^{-1} was adjusted in PBS. To perform the adherence assay, 0.5 ml of the washed epithelial cells were incubated in a glass tube with 0.5 ml of washed *C. albicans* cells in PBS at a concentration of 1×10^6 ml^{-1} in a shaking incubator at 37°C for one hour; for control, 0.5 ml of BECs were mixed with 0.5 ml of PBS. Following incubation, the cells were vacuum filtered through prewet 20-mm diameter polycarbonate filters with 12 mm pore size (Costar, MA, USA) mounted on a filter manifold (Millipore, Bedford, MA). Each filter was washed 10 times with PBS to remove unattached candidal cells. The washed filters were then removed and pressed gently onto glass slides. The slides were air-dried, heat fixed for 1 min, and Gram stained. The slides were examined under light microscopy in 1-mm intervals, and the number of candidal cells attached to 100 BECs was counted. Each experiment was performed at least two separate occasions, using BECs harvested from the same individuals at the same time on successive days; on each occasion, the experiments were performed in duplicate.

Murine model of disseminated candidiasis

Seven-week-old, male ICR mice (Harlan-Sprague) were inoculated by intravenous injection of the lateral tail vein with 1×10^6 cells of *C. albicans* strains in 0.2 ml of sterile water. Mice were followed until they were moribund, at which point they were sacrificed, or for 30 days. Survival curves were calculated according to the Kaplan-Meier method using the PRISM program (GraphPad Software) and compared using the Newman Keuls analysis. A *P*-value of <0.05 was considered significant.

Acknowledgements

We are grateful to Jeannine Brady, Paul Gulig, Shougang Jin and Bradley Bender for reviewing earlier versions of the manuscript. We thank Jill Verlander-Reed, director of the University of Florida College of Medicine Electron Microscopy

Facility, for her assistance with the immunohistochemistry experiments. William Fonzi of Georgetown University kindly provided *C. albicans* strains SC5314, CAF2-1 and CAI4. This project was supported by the American Lung Association of Florida and the National Institute of Dental and Craniofacial Research [NIH-RO1-DE13980 (M.H.N., C.J.C) and NIH-RO1-DE13523 (M.H., J.H. and A.P.F.)]. In addition, Dr Nguyen is the recipient of a V.A. Advanced Research Career Development Award. Dr Clancy is also supported by the National Institute of Allergy and Infectious Diseases (1KO8AI101758-01). Dr Cheng is a recipient of the Elizabeth Glaser Pediatric AIDS Foundation Scholar Award. The research was conducted in laboratories at the North Florida/South Georgia V.A. Medical Center, Gainesville, FL.

Sequence data for *Candida albicans* was obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. Additional sequence data was obtained from the Galar Fungail Consortium's Candida DB website set up by the Institut Pasteur (Paris, France) at http://genolist.pasteur.fr/CandidaDB/. Sequence data for *Saccharomyces cerevisiae* was obtained from the Stanford Genome Technology Center's Saccharomyces genome database at http://genome-http://www.stanford.edu/Saccharomyces/.

Note added in proof

Since the acceptance of this manuscript, *C. albicans CDC24* has been demonstrated to be required for invasive hyphal growth and full pathogenicity [Bassilana, M., Blyth, S., Arkowitz, R. A. (2003) Cdc24, the GDP-GTP exchange factor for Cdc42, is required for invasive hyphal growth of *Candida albicans*. *Eukaryot Cell* **2:** 9–18.].

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